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Reversible inhibition of caspase-3 activity by iron(III) Potential role in physiological control of apoptosis

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Abstract **Desferoxamine is known to induce apoptosis in cancer cells, but the mechanisms are still not fully understood. We have shown that iron(III) is a potent caspase-3 inhibitor, and the inhibition is reversible by the iron chelating agent desferoxamine. Also, protein disulfide isomerase (PDI) is capable of activating caspase-3 inhibited by iron(III), likely by formation of iron–sulfur complex through its active site thiols. Data presented here suggests that iron(III) could be a potential inhibitor of apoptosis in vivo, by caspase-3-dependent inhibition with a possibility of recovery through PDI overexpression.**

Keywords: Caspase-3; Protein disulfide isomerase; Iron; Desferoxamine

1. Introduction

Apoptosis is a highly regulated process of cell death. Loss of apoptotic regulation is closely linked to human diseases including various forms of cancer, ischemic damage, neurodegenerative diseases and immune disorders [1]. One of the key players in the process of apoptosis is caspase-3. Caspase-3 belongs to the family of cysteine-aspartate proteases, involved in the execution pathway of apoptosis [2,3]. Under normal cellular conditions caspase-3 exists in inactive zymogen form. Upon oxidative stress, procaspase-3 is autoproteolytically cleaved resulting in the formation of p20 and p10 domains [4] leading to assembly of a homodimer of heterodimers which represents active form of the enzyme [5]. Once activated, caspase-3 is responsible for proteolytic cleavage of a large number of substrates containing common Asp-Glu-Val-Asp motif such as PARP [6], U1-70 kDa [7], DNA-PK$_{CS}$ [8], PKC δ [9], Huntington [10] and SREBP-1 [11].

Previous in vitro and in vivo studies have shown that the active-site cysteine residue is susceptible to oxidative modification which results in caspase-3 inactivation and inhibition of apoptosis. Nitric oxide (NO) can act as a reversible inhibitor of caspase-3 activity, by nitrosating its active site cysteine [12,13]. Reactive oxygen species such as hydrogen peroxide (H_2O_2) reversibly inhibit caspase-3 activity [14]. Recent discovery of metal ion-dependent regulation of apoptosis suggested new mechanisms for caspase-3 inhibition [15,16].

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Iron is an essential component of various biochemical processes including cell proliferation, electron transfer and detoxification [17]. Intracellular iron trafficking occurs through the cell surface transferrin receptor (TfR) [18,19]. While the expression of transferrin receptor in non-dividing cells is low, carcinoma cell lines are associated with overexpression of the iron receptor [20]. Consequently, increased iron levels have been observed in various cancer types [21] including HL-60 leukaemic cells, human breast cancer cells and human neuroblastoma cells as detected by apoptosis markers amongst which is an increased caspase-3 activity. However, there has been no direct evidence of caspase-3 interaction with iron.

Protein disulfide isomerase (PDI) is a multifunctional enzyme expressed throughout the cell [22,23]. PDI is an abundant protein [35] found in various cell compartments including ER, cell surface, the cytosol and the nucleus [for review see [36]]. The main function of PDI is isomerization and rearrangement of disulfide bonds [24]. The active site of PDI contains two vicinal thiols that undergo redox state changes during catalysis [25]. Recent reports showed that PDI has zinc [26] and copper [27] binding activity through its active site thiols. PDI active site resembles [2Fe–2S]-ferrodoxin iron biding domains [28] suggesting that it may bind iron as well.

Here, we present a study on the effects of iron on caspase-3 activity and on the role of PDI in reversing this effect.

2. Materials and methods

2.1. Protein expression and purification

PDI was expressed and purified as described in Sliskovic et al. [29]. Protein quantification and purity was preformed using the Bradford assay and gel electrophoresis/Western blotting, respectively.

Active caspase-3 was expressed in *E. coli* BL21(DE3) with the expression vector pET-28a as previously described [30]. Purification of caspase-3, protein quantification and purity was carried out as described in the above paragraph.

2.2. Caspase-3 activity

The conditions used for assays of caspase-3 activity are specified in figure legends. Caspase-3 activity was evaluated by measuring proteolytic cleavage of fluorogenic synthetic substrate Ac-DEVD-AMC. Assay buffer contained 100 mM phosphate buffer, pH 7.0, unless stated otherwise. When needed, 100 mM phosphate buffer, pH 7.0, was prepared in MiliQ water (MilliQ synthesis, Millipore) and treated in Chelex-100 resin (Bio-Rad) to obtain iron-free phosphate buffer. Remaining iron was quantified as described below. Fluorescence increase due to AMC release was monitored using 360 nm excitation and 460 nm emission wavelengths. All caspase-3 activity experiments were performed using Wallac Victor^{3TM} 1420 Multilabel Counter (Perkin–Elmer) and Varian Cary Eclipse fluorescence spectrometer.

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2.3. Preparation of PDI-bound iron

PDI (1 mg) was incubated with 5 × molar excess of $Fe_2(SO4)_3$ for 1 h at room temperature in 100 mM phosphate buffer, pH 7.0. Following incubation, excess iron was removed over Sephadex G-25 with 100 mM phosphate buffer, pH 7.0. Final PDI concentration was quantified using Bradford assay. PDI–Fe was processed immediately for iron determination.

2.4. Iron determination

Quantification of complexed iron was done as described by Fish [31], using Agilent UV–visible spectrophotometer.

2.5. Chemical blocking of PDI thiols

PDI active site thiols were chemically modified using *N*-ethyl maleimide (NEM) and phenylarsine oxide (PAO). Prior to chemical blocking, PDI (1 mg) was incubated with 5× molar excess dithiothreitol (DTT) for 2 h at the room temperature to assure full reduction of the active site thiols. Excess DTT was removed over Sephadex G-25 using 100 mM phosphate buffer, pH 7.0. Reduced PDI was immediately incubated with 5× molar excess NEM and PAO for 2 h at the room temperature and chromatographed over Sepadex G-25 column. Thiol blocking was confirmed with DTNB assay.

3. Results

Assay conditions for measuring caspase-3 activity in the literature generally employ chelating agents such as EDTA, as well as reducing agent DTT in order to maintain the active site cysteine in its reduced form [32]. We first sought to determine the kinetic parameters of recombinant caspase-3 in the presence and absence of chelating and reducing agents. EDTA totally abolished the need for employing reducing agents (DTT or GSH) (Supporting Material, Figure S1 A) indicating that the inactivation was due to contaminating metal ions.

In the absence of EDTA (Supporting Material, Figure S1 B), DTT was more successful in activating caspase-3 than GSH. The apparent KM of caspase-3 was found to be ~20 ± 2 μM in the presence of EDTA (±DTT, GSH) (Figure S1 A) and ~15 ± 2 μM in the absence of EDTA (+DTT) (Figure S1 B). In order to identify the metal ions responsible for caspase-3 inhibition, the activity of the enzyme was measured in the presence of different metal ion chelators. As shown in Fig. 1, besides EDTA, desferoxamine (DFO, 100 μM) was more successful than neocuproine in restoring caspase-3 (25 nM) activity. DFO has the highest affinity for Fe(III). Therefore, the results obtained here suggested that iron is responsible for inhibiting caspase-3 activity. To further test this hypothesis the activity of caspase-3 was measured in metal free buffer (Fig. 2). As expected caspase-3 was active in the absence of metal ions. Upon addition of 1–100 μM Fe(III) to 25 nM enzyme, ~90 % of the activity was lost. The IC_{50} estimated for Fe(III)-mediated inhibition of caspase-3 was ~7 ± 2 μM.

Next, we investigated the possibility of PDI to act as a metal chelator since its active site with the CXXC motif is similar to [2Fe–2S]-ferrodoxin iron biding domains. To this end caspase-3 (25 nM) incubated in phosphate buffer (100 mM, pH 7.0) resulted in no detectable activity. To this, increasing amounts of PDI (25 nM to 2.5 μM) were added. The result was a PDI-dependent increase in caspase-3 activity (Fig. 3A). In similar experiments caspase-3 activity was monitored in the phosphate buffer (Fig. 2B (◆)). Addition of DFO (100 μM) gave rise to an initial rate v_0 of 0.3 μM/min (Fig. 2B (◇)). In the presence of PDI (1 μM) the v_0 was ~2.5-fold larger that observed with DFO alone (Fig. 2B (■)). Caspase-3 activity in the presence of both PDI DFO gave a v_0 that was ~14% lower than that observed with PDI alone (Fig. 3B (□)). These results suggest that PDI is a better Fe(III) chelator than DFO and raises the possibility that DFO bound Fe(III) can still bind to the caspase-3 active site and partially inactivating it.

In a further attempt to demonstrate that the PDI-mediated activation of caspase-3 was related to Fe(III), PDI (1 μM) and caspase-3 (25 nM) were added to metal ion free buffer and the initial rates of DEVD-AMC cleavage was monitored

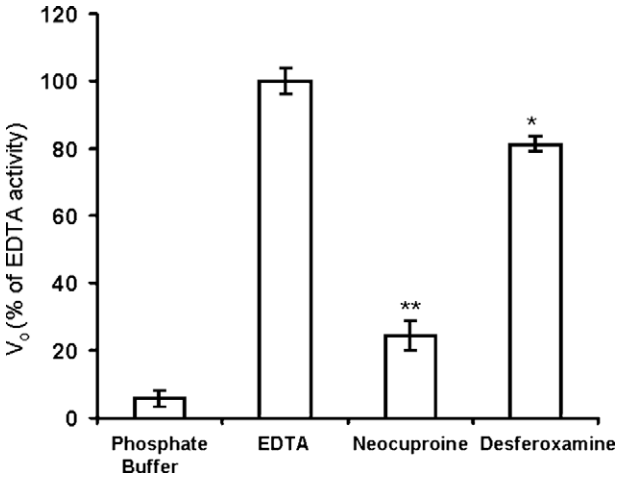

Fig. 1. Chelating agent dependent activation of caspase-3. Caspase-3 (25 nM) activity was monitored over 15 min in the presence of Ac-DEVD-AMC (20 μM). Assay buffer contained 100 mM phosphate, pH 7.0, EDTA (100 μM), neocuproine (100 μM) or desferoxamine (100 μM). Final rates were obtained by correcting for the blank rates without caspase-3 and represented as the percentage of the highest rate. ($^{*}P = 0.01$ compared to EDTA, $^{**}P = 0.001$ as compared to phosphate buffer.)

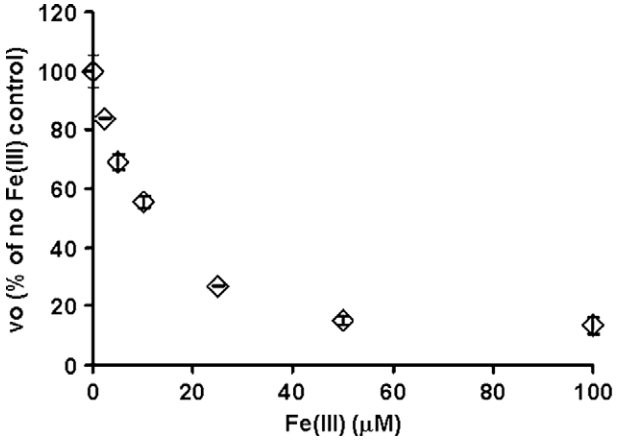

Fig. 2. Iron dependent inhibition of caspase-3 activation. Caspase-3 (25 nM) activity was assessed by Ac-DEVD-AMC (20 μM) in the presence of increasing iron concentrations (1–100 μM) in the iron-free (<50 nM as established by the iron determination as described in Section 2) phosphate buffer. The initial rates were measured over 15 min and corrected for the corresponding controls without caspase-3. The rates are represented as the percent of caspase-3 without iron.

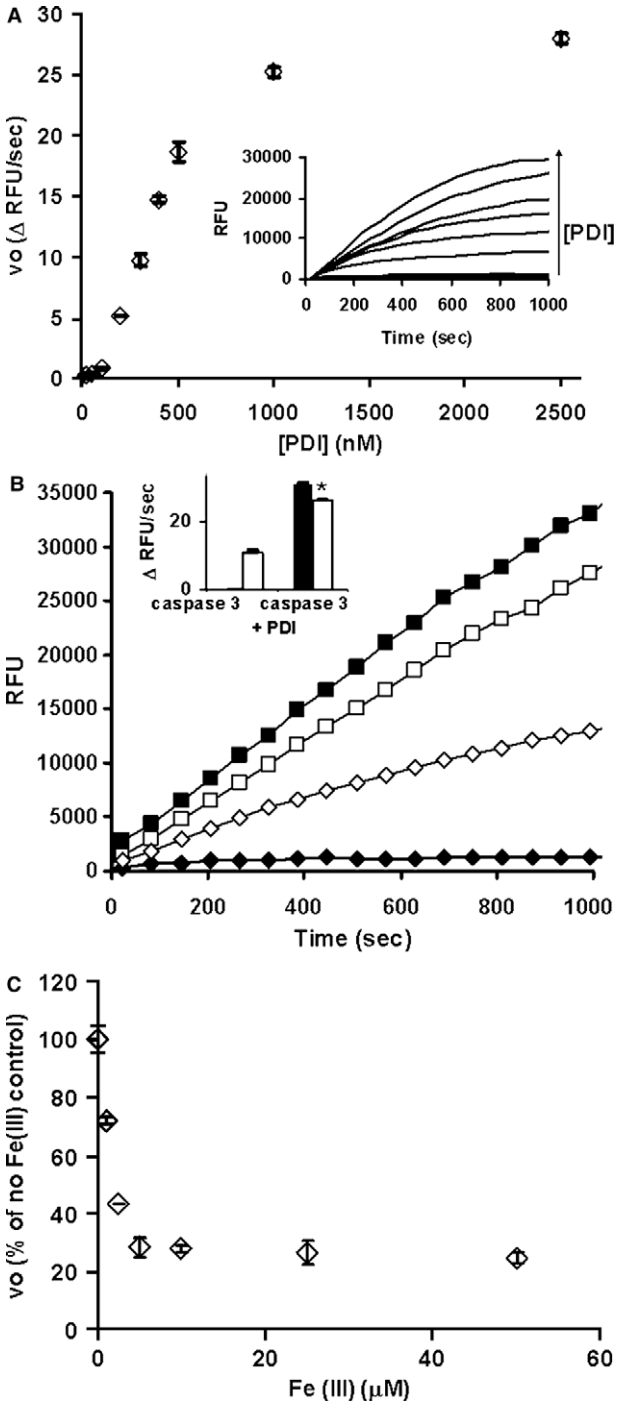

Fig. 3. PDI dependent caspase-3 activation. (A) Initial rates of caspase-3 activation were monitored in the presence of increasing PDI concentrations (25–2500 nM), using Ac-DEVD-AMC (20 μM, over 15 min (inset). Final rates due to presence of PDI were obtained by subtracting the blank which contained PDI only. (B) The role of PDI (1 μM) dependent caspase-3 (25 nM) activation was measured in the presence (white symbols) and absence (black symbols) of DFO (100 μM), using Ac-DEVD-AMC (20 μM) as the substrate. Traces shown are representative of caspase-3 activity in the phosphate buffer without (◆) and with PDI (■), and DFO containing buffer without (◇) and with PDI (□). The initial rates of caspase-3 activation in the phosphate buffer are represented by black bars, and in the presence of DFO by white bars (inset). ($^{*}P = 0.001$). (C) Caspase-3 (25 nM) activity was monitored by Ac-DEVD-AMC (20 μM) in the presence of PDI (1 μM), and increasing iron concentrations (1–100 μM). The initial rates of caspase-3 activity were monitored over 15 min, and subtracted for the control which did not contain caspase-3.

as a function of [Fe(III)] (1–100 μM). Under these conditions PDI-activated caspase-3 activity decreased by ~80% in an [Fe^{3+}]-dependent manner with an apparent IC_{50} of ~3 ± 1 μM.

Next we asked if iron-dependent caspase-3 inhibition was a reversible process. Initial rate of DEVD-AMC cleavage due to freshly prepared caspase-3 (25 nM) was inhibited by the addition of iron (50 μM) (Fig. 4A) and the activity was successfully restored by the addition of DFO (100 μM). Similarly, caspase-3 inhibited by the addition of iron could be reactivated by the addition of either DTT (1 mM) (Fig. 4B, black trace) or PDI (2 μM) (Fig. 4B, grey trace). These results prompted us to investigate whether active site thiols are essential for PDI-mediated caspase-3 activation. Caspase-3 activation was monitored in the presence of native PDI and PDI whose active site thiols were blocked with NEM. Results obtained demonstrate that modification of PDI active site by NEM prevents caspase-3 activation (Fig. 5). In light of these results, the ability of native PDI to bind iron was tested. PDI pretreated with iron was found to bind ~2 moles of iron per mole of active site thiols (Fig. 6).

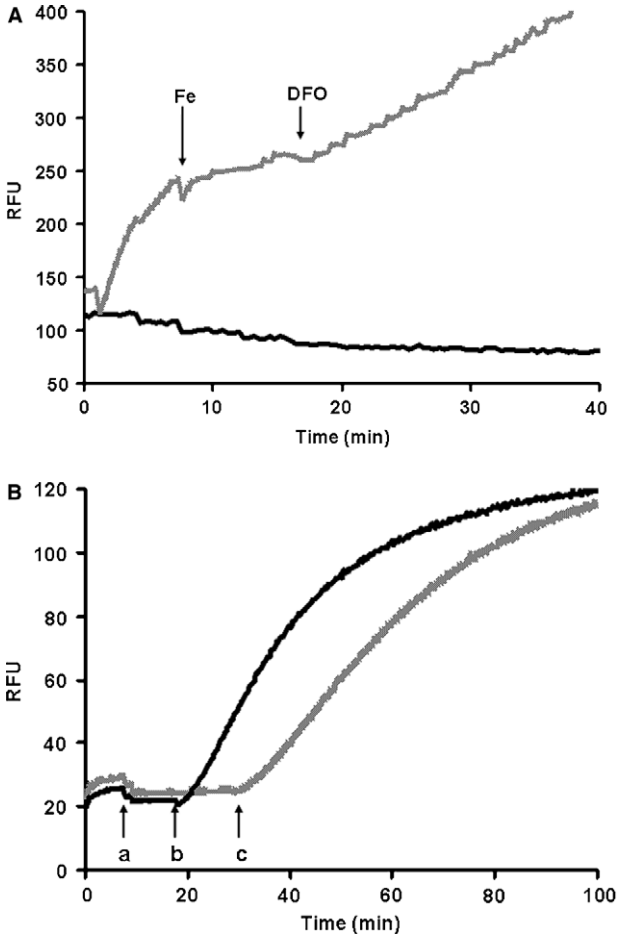

Fig. 4. Reversible activation of iron-inhibited caspase-3. (A) Initial caspase-3 (25 nM) activity in the iron-free phosphate buffer, pH 7.0, was inhibited by addition of iron (50 μM), and followed by reactivation with DFO (100 μM) (grey trace). Blank (black trace) did not contain any caspase-3 ($n = 3$). (B) Caspase-3 (25 nM) activity was inhibited by addition of iron (50 μM) (a), followed by reactivation with DTT (1 mM) (black trace, b) and PDI (2 μM) (grey trace, c), ($n = 3$).

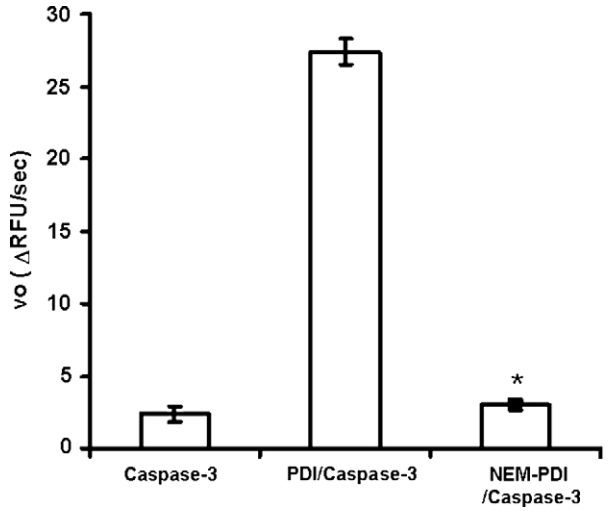

Fig. 5. The role of PDI active site thiols in C3 activation. Caspase-3 (25 nM) activity was monitored in the absence and presence of PDI and NEM-blocked PDI (1 μM) over 15 min, using Ac-DEVD-AMC (20 μM). NEM-blocked PDI was prepared as outlined in Section 2. ($P < 0.1$).

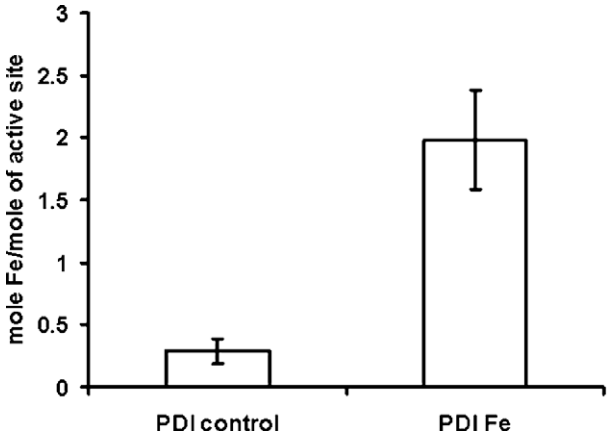

Fig. 6. PDI-bound iron estimation. Iron estimation was performed for the PDI samples without iron (PDI control) and with iron incubation (PDI-Fe). Assay was carried out as described in Section 2.

4. Discussion

We have demonstrated that iron(III) can inhibit caspase-3 activity. Activity is restored in the presence of a specific iron(III) chelating agent DFO (Fig. 1) and interestingly, in the presence of PDI (Fig. 3A–C). In addition, PDI appeared to be better at chelating iron than DFO in that 100-fold lower [PDI] was able to yield ~2-fold larger activity from fully iron-inactivated caspase. We believe this difference arises because the DFO–Fe(III) complex still can interact with the caspase active site and partially block its activity. On the other hand, this is not possible with the much larger PDI–Fe(III) complex (Fig. 3 B). PDI dependent caspase-3 activation was attenuated by the addition of iron (Fig. 3C) with an estimated IC_{50} of ~3 μM. These results provided a direct evidence of caspase-3 inhibition by Fe(III), which supports previous observations that iron chelating agents can induce apoptosis in various cancer cell lines.

To date, NO and H_2O_2 has been shown to reversibly inactivate caspase-3 [13,14,37]. In this study, we were able to show that iron(III) can also reversibly regulate caspase-3 activity most likely by interacting with active site Cys^{285} since the inactivation is reversible by thiol reductants (Figure S1) as well with iron chelators. In addition our data does not preclude iron interacting at allosteric sites elsewhere in the molecule. A role for iron(III) in inactivating caspase-3 was established by the effect of the Fe(III) specific chelator DFO on restoring caspase-3 activity (Fig. 4A) and by the direct inhibition of caspase and PDI-activated caspase by iron(III) (Figs. 2 and 3C, respectively). The fact that the inhibition by iron(III) is reversible with DFO (and PDI) (Fig. 4A and B, respectively) suggests that iron does not play a role in altering the oxidation state of the active site Cys^{285}. In addition to DFO, ubiquitous enzyme PDI was able to mimic the actions of DFO in restoring caspase-3 activity at physiologically relevant concentrations. Our data demonstrates that active site thiols of PDI are essential for caspase-3 activation (Fig. 5). Previous reports demonstrated that PDI-like proteins such as glutaredoxin are able to form iron–sulfur complexes [33]. PDI was able to bind iron at a 2:1 iron:cysteine thiol ratio suggesting that PDI most likely binds Fe(III) through its active site thiols, as it was shown to bind Zn and Cu.

Based on our findings, we are proposing a physiological role for both Fe(III) and PDI in the modulation of caspase-3 activity and in the process regulating apoptosis. Previous studies showed a correlation between cancer cells and increased iron levels due to (i) increased expression of TfR [20], (ii) increased ferritin (iron storage) secretion [21] and (iii) ability of desferoxamine (DFO) to induce apoptosis in cancer cells [17]. Here, we show that a potential target of Fe(III) is caspase-3, a central regulator of apoptosis. Therefore, we propose the mechanism illustrated in Fig. 7 for the regulation of apoptosis by Fe(III) and PDI. Cells upon becoming cancerous, upregulate TfR resulting in larger intracellular levels of Fe(III). The Fe(III)

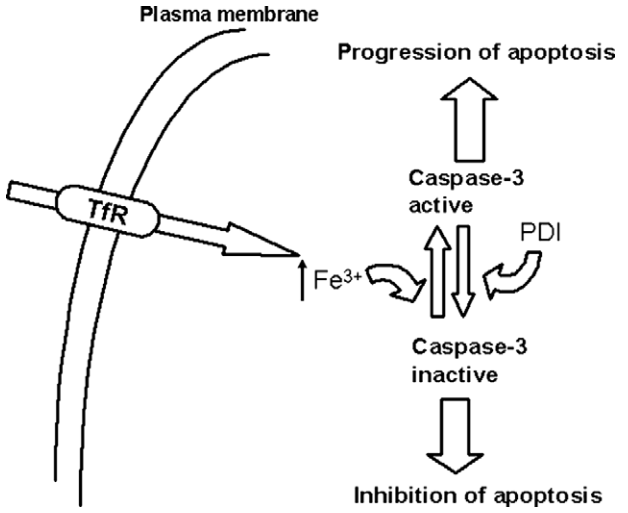

Fig. 7. Proposed mechanism of caspase-3 regulation in cancer cells. Cancer cells are associated with overexpression of TfR resulting in increased levels of intracellular [Fe^{3+}] which could lead to caspase-3 inhibition and suppression of apoptosis. Overexpression of PDI could restore caspase-3 activity, enabling apoptosis to proceed.

inactivates caspse-3 thus arresting apoptosis. In cells, that successfully upregulate and secrete PDI, blocking of caspase-3 activity by Fe(III) would result in the re-initiation of apoptosis and cell death. These results suggest that substances that induce PDI overexpression [34] could potentially be employed as new anti-cancer therapeutics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.03.031.

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